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01/13/99

09/229324

01/13/99

Date: January 13, 1999

Docket No.: 760-262P

Assistant Commissioner for Patents  
Washington, D.C. 20231

Sir:

This is a Request for filing a X continuation        divisional application under 37 C.F.R. § 1.53(b) of pending prior Application No. 08/668,464 filed on June 25, 1996, the entire contents of which are hereby incorporated by reference, by

Yukoh HIEI

Toshihiko KOMARI

For

METHOD FOR TRANSFORMING MONOCOTYLEDONS

1. ✓ X Enclosed is an application consisting of specification, claims, declaration and drawings/photographs (if applicable).
2. X The filing fee has been calculated as follows:

Continuation Application  
under 37 C.F.R. § 1.53(b)  
of Application No. 08/668,464  
Attorney Docket No. 760-262P

		LARGE ENTITY	SMALL ENTITY
BASIC FEE		\$760.00	\$380.00
NUMBER FILED	NUMBER EXTRA	RATE FEE	RATE FEE
TOTAL CLAIMS	42- 20 =	x 18 = \$396.00	x 9 = \$
INDEPENDENT CLAIMS	2- 3 =	x 78 = \$	x 39 = \$
MULTIPLE DEPENDENT <u>X</u> CLAIMS PRESENTED		+ \$260.00	+ \$130.00
TOTAL		\$1,416.00	

3. X A check in the amount of \$1,416.00 to cover the filing fee and recording fee (if applicable) is enclosed.
4.      Please charge Deposit Account No. 02-2448 in the amount of \$          . A triplicate copy of this request is enclosed.
5. X Amend the specification by deleting lines 4-9 of page 1 and inserting the following:
- This application is a continuation of copending Application No. 08/668,464, filed on June 25, 1996, which is a continuation-in-part of Application No. 08/193,058 which is the national phase of PCT International Application No. PCT/JP93/00925 filed on July 6, 1993 under 35 U.S.C. § 371 (now U.S. Patent No. 5,591,616 issued on January 7, 1997). The entire contents of each of the above identified applications are hereby incorporated by reference.--
6.      Transfer the drawings/photographs from the prior application to this application and abandon said prior application as of the filing date accorded this application. A duplicate copy of this request is enclosed for filing in the prior application file.
7. X Enclosed are two (2) sheet(s) of formal drawings/photographs.
8.      A verified statement claiming small entity status was filed in prior Application No.                                  on     . See attached copy of verified statement claiming small entity.

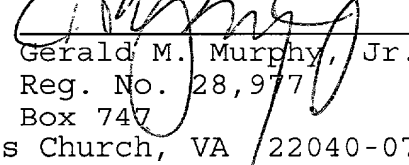
08/668,464

9. X The prior application is assigned to  
JAPAN TOBACCO INC.
10. ✓X A Preliminary Amendment is enclosed.
- 11a.     Priority of Application No.            filed in        on  
                   is claimed under 35 U.S.C. § 119. See  
attached copy of the Letter claiming priority filed in  
the prior application on                   .
- 11b. X Priority of International Appln. PCT/JP93/00925 filed  
on July 6, 1993 under the Patent Cooperation Treaty and  
Application No. 4-204464 filed in Japan on July 7, 1992  
under 35 U.S.C. § 119 are hereby reclaimed.
12. ✓X An Information Disclosure Statement and PTO-1449  
form(s) are attached hereto for the Examiner's  
consideration.
13. X Address all future communications to:  
  
BIRCH, STEWART, KOLASCH & BIRCH, LLP  
P.O. Box 747  
Falls Church, Virginia 22040-0747  
  
Telephone: (703) 205-8000
14. X An extension of time for three (3) month(s) January 13,  
1999 has been submitted in parent Application No  
08/668,464 in order to establish copendency with the  
present application.
15.     Also enclosed herewith is the following:

If necessary, the Commissioner is hereby authorized in this,  
concurrent, and future replies, to charge payment or credit any  
overpayment to Deposit Account No. 02-2448 for any additional fees  
required under 37 C.F.R. § 1.16 or under 37 C.F.R. § 1.17;  
particularly, extension of time fees.

Respectfully submitted,

BIRCH, STEWART, KOLASCH & BIRCH, LLP

By:   
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GMM/MAL/cpw

IN THE U.S. PATENT AND TRADEMARK OFFICE

APPLICANT: Yukoh HIEI et al.  
SERIAL NO.: Continuation Application  
under 37 C.F.R. § 1.53(b) of  
Application No. 08/668,464 GROUP NO.: 1649  
FILED: January 13, 1999 EXAMINER: Benzion  
FOR: METHOD FOR TRANSFORMING MONOCOTYLEDONS

PRELIMINARY AMENDMENT

Assistant Commission for Patents  
Washington, D.C. 20231  
Sir:

January 13, 1999

The following preliminary amendments and remarks are respectfully submitted in connection with the above-identified application.

IN THE CLAIMS:

Please amend the following claims:

Claim 1.

Line 6, replace "less than 7" with --1-6--.

Claim 13.

Line 26, replace "less than 7" with --1-6--.

REMARKS

Entry of the above amendments is earnestly solicited. An early and favorable first action on the merits is earnestly solicited.

Should the Examiner have any questions regarding the present application, he is requested to contact MaryAnne Liotta, PhD (Reg. No. 40,069) in the Washington DC area, at (703) 205-8000.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. §§1.16 or 1.17; particularly, extension of time fees.

Respectfully submitted,

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760-262P

## SPECIFICATION

## Method for Transforming Monocotyledons

CROSS-REFERENCE TO THE RELATED APPLICATIONS

This is a Continuation-in-Part of copending U.S.  
5 Application Serial No. 08/193,058 filed on May 3, 1994,  
which claims priority under the Patent Cooperation Treaty  
on International Application No. PCT/JP93/00925 filed on  
July 6, 1993. The entire contents of both of these  
applications are hereby incorporated by reference.

10

Technical Field

The present invention relates to a method for  
transforming monocotyledons.

Background Art

Conventional methods for transforming monocotyledons  
15 include electroporation method, polyethylene glycol  
method (PEG method), particle gun method and so on.

The electroporation method is a method in which  
protoplasts and the desired DNA are mixed, and holes are  
formed in the cell membranes by electric pulse so as to  
20 introduce the DNA into the cells, thereby transforming  
the cells. This method currently has the highest  
reproducibility of the conventional methods and various  
genes have been introduced into monocotyledons,  
especially into rice plants by this method (Toriyama K.  
25 et al., 1988; Bio/Technol. 6:1072-1074, Shimamoto K. et  
al., 1989; Nature 338:274-276, Rhodes C.A. et al., 1989;  
Science 240:204-207). However, this method has the

problems that 1) it can be applied only to the plant species for which the system for regenerating plants from protoplasts has been established, 2) since it takes several months to regenerate plants from the protoplasts, a long time is required to obtain transformants, and that 3) since the culture period is long, the frequency of emergence of mutants during the culture is high accordingly, so that the probability of obtaining normal transformants is decreased.

10       The PEG method is a method in which the desired gene and protoplasts are mixed and the mixture is treated with PEG, thereby introducing the gene into the protoplasts. This method is different from the electroporation method in that PEG is used instead of the electric pulse. The efficiency of introducing the gene is thought to be somewhat lower than the electroporation method. Although there is a report that transformants were obtained by this method, this method is not widely used. Since protoplasts are used, this method has the same problems as in the electroporation method (Zhang W. et al., 1988; Theor. Appl. Genet. 76:835-840, Datta S.K. et al., 1990; Bio/Technol. 8:736-740).

25       The particle gun method is a method in which the desired gene is attached to fine metal particles, and the metal particles are shot into cells or tissues at a high speed, thereby carrying out the transformation. Thus, according to this principle, transformation may be

performed on any tissues. Therefore, this method is effective for transforming the plant species for which the systems for regenerating plants from protoplasts have not been established. The efficiency of transformation varies depending on the selection after the gene was shot. There is no data which compare the efficiency of this method with that of the electroporation method (Gordon-Kamm W.J. et al., 1990; Plant Cell 2:603-618, Fromm M.E. et al., 1990; Bio/Technol. 8:833-839, Christou P. et al., 1991; Bio/Technol. 9:957-962).

Other methods include 1) culturing seeds or embryos with DNA (Topfer R. et al., 1989; Plant Cell 1:133-139, Ledoux L. et al., 1974 Nature 249:17-21); 2) treatment of pollen tube (Luo and Wu 1988; Plant Mol. Biol. Rep. 6:165-), 3) liposome method (Caboche M. 1990; Physiol. Plant. 79:173-176, Gad A.E. et al., 1990:177-183) and 4) microinjection method (Neuhaus G. et al., 1987; Theor. Appl. Genet. 75:30-36). However, these methods have problems in the efficiency of transformation, reproducibility or applicability, so that these methods are not commonly used.

On the other hand, a method for introducing a gene using the Ti plasmid of bacteria belonging to genus Agrobacterium as a vector is widely used for transforming dicotyledons such as tobacco, petunia, rape and the like. However, it is said that the hosts of the bacteria belonging to genus Agrobacterium are restricted to



dicotyledons and that monocotyledons are not parasitized by Agrobacterium (De Cleene M. 1976; Bot. Rev. 42:389-466).

As for transformation of monocotyledons by Agrobacterium, although transformation of asparagus (Bytebier B. et al., 1987: Proc. Natl. Acad. Sci. USA, 84:5345-5349) and of Dioscorea bulbifera (Schafew et al., 1987; Nature 327:529-532) has been reported, it is said that this method cannot be applied to other monocotyledons, especially to the plants belonging to family Gramineae (Potrykus I. 1990; Bio/Technol. 8:535-543).

Grimsley et al. (1987: Nature 325:177-179) reported that T-DNA of Agrobacterium in which DNA of maize streak virus was inserted was inoculated to the apical meristem of maize plants and infection of the plants by maize streak virus was confirmed. Since the infected symptoms are not observed when merely the DNA of maize streak virus is inoculated, they interpreted the above-mentioned result as a piece of evidence showing that Agrobacterium can introduce DNA into maize. However, since it is possible that a virus replicates even if it is not incorporated into the nucleus genome, the result does not show that the T-DNA was incorporated into the nucleus. They subsequently reported that the infection efficiency is the highest when the virus is inoculated to the apical meristem in the shoot apex of the maize (Grimsley et al.,

1988: Bio/Technol. 6:185-189), and that virC gene in the plasmid of Agrobacterium is indispensable to the infection (Grimsley et al., Mol. Gen. Genet. 217:309-316).

Gould J. et al. (1991; Plant Physiol. 95:426-434)  
5 inoculated super-virulent Agrobacterium EHA1 having a kanamycin-resistant gene and a GUS gene to shoot apices of maize after injuring the shoot apices with a needle, and selected the shoot apex based on the resistance to kanamycin. As a result, plants having resistance to  
10 kanamycin were obtained. They confirmed by Southern blotting analysis that some of the seeds of the subsequent generation of the selected plants had the introduced gene (chimera phenomenon).

Mooney P.A. et al., (1991; Plant Cell, Tissue, Organ  
15 Culture 25:209-218) tried to introduce kanamycin-resistant gene into embryos of wheat using Agrobacterium. The embryos were treated with an enzyme to injure the cell walls, and then Agrobacterium was inoculated. Among the treated calli, although very small  
20 number of calli which were assumed to be transformants grew, plants could not be regenerated from these calli. The existence of the kanamycin-resistant gene was checked by Southern blotting analysis. As a result, in all of the resistant calli, change in structure of the  
25 introduced gene was observed.

Raineri et al. (1990; Bio/Technol. 8:33-38)  
inoculated super-virulent Agrobacterium A281 (pTiBo542)

to 8 varieties of rice after injuring the scutella of the rice plants. As a result, growth of tumor-like tissues was observed in two varieties, Nipponbare and Fujisaka 5. Further, an Agrobacterium containing a plasmid having a T-DNA from which a hormone-synthesizing gene was removed and instead, a kanamycin-resistant gene and GUS gene were inserted therein was inoculated to embryos of rice. As a result, growth of kanamycin-resistant calli was observed. Although the expression of GUS gene was observed in these resistant calli, transformed plants could not be obtained from the calli. They interpreted these results as that the T-DNA was introduced into rice cells.

Thus, although the experimental results which suggest that introduction of genes into the plants belonging to family Gramineae such as rice, maize and wheat can be attained by using Agrobacterium have been reported, fully convincing results have not been obtained about the reproducibility, introduction efficiency and about the confirmation of the introduction of the gene (Potrykus I. 1990; Bio/Technol. 8:535-543).

As mentioned above, introduction of genes into the plants belonging to family Gramineae is now mainly carried out by the electroporation method. However, with this method, since protoplasts are used, a long time and much labor are required to obtain regenerated plants. Further, there is a danger that mutants may emerge at a high frequency due to the long culturing period. Still

further, this method cannot be applied to the plants such as maize for which the system for regenerating plants from protoplasts has not been established. In view of this, as mentioned above, as for maize, it has been tried  
5 to use the apical meristem. However, the operation for isolating the apical meristem requires much labor and it is not easy to prepare apical meristem in a large amount.

#### Disclosure of the Invention

Accordingly, an objective of the present invention  
10 is to provide a method for transforming monocotyledons, with which the time required for obtaining regenerated plants from the time of transformation is shorter than that in the conventional methods, which can be generally applied even to the plants for which the systems for  
15 regenerating plants from protoplasts have not yet been established, and with which the preparation of the materials to be used is easy.

The present inventors intensively studied the influences of the monocotyledonous plant tissues treated  
20 with Agrobacterium, treatment conditions of Agrobacterium, constitution of the binary vector and the like on the introduction efficiency of the genes to discover that cultured tissues of monocotyledons can be transformed by using Agrobacterium with drastically high efficiency and  
25 reproducibility and that by employing this method, the above-mentioned object can be attained, thereby completing the present invention.

That is, the present invention provides a method for transforming a monocotyledon, comprising contacting a cultured tissue of said monocotyledon during dedifferentiation thereof obtained by culturing an explant on a dedifferentiation-inducing medium for less than 7 days with a bacterium belonging to the genus *Agrobacterium* containing a super binary vector having the virulence region of Ti plasmid pTiBo542 contained in *Agrobacterium tumefaciens* A281, left and right border sequences of T-DNA of a Ti plasmid or an Ri plasmid of a bacterium belonging to the genus *Agrobacterium*, and a desired gene located between said left and right border sequences. The present invention also provides a method for transforming a monocotyledon, comprising contacting a cultured tissue of said monocotyledon during dedifferentiation thereof obtained by culturing an explant derived from an immature tissue on a dedifferentiation-inducing medium for less than 7 days with a bacterium belonging to the genus *Agrobacterium* containing a desired gene and containing a vector having the virulence region of Ti plasmid contained in *Agrobacterium tumefaciens*.

By the present invention, it was first attained to introduce foreign genes to monocotyledons such as plants belonging to family Gramineae including rice, maize, wheat, barley and the like with good reproducibility. Although methods for transforming monocotyledons using

Agrobacterium are known, they are not established methods as mentioned above. In contrast, according to the present invention, by inoculating Agrobacterium to cultured tissues which have not been employed in the conventional methods, genes can be very easily introduced. In the present invention, since a cultured tissue such as a callus which can be easily prepared is used, the sample materials can be obtained more easily than the conventional method employing the apical meristem. Further, since cultured cells are transformed, the time required for regenerating plants is shorter than in cases where protoplasts are transformed, so that the frequency of mutation is decreased. Further, by employing a super binary vector, it was first attained to introduce genes with high efficiency into varieties which are difficult to culture such as a variety of rice. Still further, as will be described in the examples below, by employing an appropriate selection method after inoculation, the chimera phenomenon in which the desired gene is introduced chimerally can be decreased.

#### Brief Description of the Drawings

Fig. 1 shows the structure of pTOK162 which is an example of the plasmid contained in Agrobacterium bacteria that may be employed in the method of the present invention, and shows the method for constructing a plasmid pTOK232 used in the examples of the present invention; and

Fig. 2 shows the method for constructing a plasmid pTOK233 used in the examples of the present invention, which plasmid is similar to pTOK232.

Best Mode for Carrying Out the Invention

5           The monocotyledons which may be transformed by the method according to the present invention are not restricted and the method according to the present invention may be applied to any monocotyledons such as rice, maize, barley, wheat, asparagus and the like.

10           The cultured tissue used in the method of the present invention is a cultured tissue during dedifferentiation process. The term "cultured tissue during the dedifferentiation process" herein means a tissue which is obtained by culturing an explant on a medium containing a plant growth regulator such as an auxin or a cytokinin for less than 7 days, preferably 1-6 days, which tissue is before the stage that a callus or an adventitious embryo-like tissue is formed. The term "dedifferentiated tissue" herein means a callus or an  
15           adventitious embryo-like tissue obtained by culturing an explant in a medium containing a plant growth regulator such as an auxin or a cytokinin. The cultured tissue employed in the present invention may be originated from any part of the plant. For example, cultured tissues  
20           originated from scutellum, shoot apex, root, immature embryo, pollen and anther can be employed. The tissue to be cultured may preferably be an immature tissue such as  
25

immature embryo. The term "immature" herein means that the tissue has not reached the matured state of the tissue and will mature under conditions which allow the maturation. Dedifferentiation-inducing media are well-known in the art. For example, a medium containing major inorganic salts and vitamins of N6 medium (Chu C.C. 1987; Proc. Symp. Plant Tissue Culture, Science Press Peking, pp.43-50), 2 mg/l of 2,4-D, 1 g/l of casamino acid, 30 g/l of sucrose and 2 g/l of Gelrite, or a medium containing inorganic salts and vitamins of LS medium (Linsmaier, E., and Skoog, F. 1965; Physiol. Plant 18:100-127), 100 mg/l of casamino acid, 700 mg/l of proline, 1.5 mg/l of 2,4-D, 20 g/l of sucrose and 2.3 g/l of Gelrite. It should be noted that the cultured tissue employed in the method of the present invention is not necessarily a callus, but suspension cells may also be employed.

As the Agrobacterium used for the transformation, Agrobacterium which are employed for the transformation of dicotyledons can be employed. Many of these Agrobacterium contain a vector having a DNA region originated from the virulence region (vir region) of Ti plasmid originated from Agrobacterium tumefaciens. The gene encoding a character which is desired to be given to the plant is inserted in this vector, or exists in a separate plasmid and inserted into the Ti plasmid in vivo by homologous recombination or the like. The present



inventors previously developed a vector containing a DNA region originated from the virulence region (vir region) of Ti plasmid pTiBo542 (Jin S. et al., 1987; J. Bacteriol. 169:4417-4425) contained in a super-virulent

5 Agrobacterium tumefaciens A281 exhibiting extremely high transformation efficiency (Hood E.E. et al., 1984; Bio/Technol. 2:702-709, Hood E.E. et al., 1986; J. Bacteriol. 168:1283-1290, Komari T. et al., 1986; J. Bacteriol. 166:88-94, Jin S. et al., 1987; J. Bacteriol.

10 169:4417-4425, Komari T. 1989; Plant Science 60:223-229 ATCC37394) (Japanese Laid-open Patent Application (Kokai) No. 4-222527. In this specification, this vector is also called "super binary vector"). That is, super binary vector is a vector having the virulence region of Ti

15 plasmid pTiBo542 contained in *Agrobacterium tumefaciens* A281, left and right border sequences of T-DNA of a Ti plasmid or an Ri plasmid of a bacterium belonging to the genus *Agrobacterium*, and a desired gene located between said left and right border sequences. Such a super

20 binary vector may preferably be employed in the present invention. The above-mentioned virulence region of the super binary vector preferably comprises *VirB* and *VirG* regions.

An example of such a super binary vector is pTOK162

25 (Japanese Laid-open Patent Application (Kokai) No. 4-222527, EP-A-504,869, U.S. Serial No. 07/854,844). The structure is shown in Fig. 1. This plasmid comprises a

plasmid called pTOK154 which can replicate in both Escherichia coli and in Agrobacterium tumefaciens (pTOK154 is a plasmid containing T region, which was constructed by the method described below from a known plasmid pGA472 derived from the Ti plasmid and a known plasmid having a wide host spectrum called pVCK101), into which a Kpn I fragment (containing virB, virG and virC genes) with a size of 15.2 kb originated from the virulence region of pTiBo542 is inserted, the Kpn I fragment having been cloned. In pTOK154, between two border sequences of the T region, a kanamycin-resistant gene is inserted as a gene to be introduced into a monocotyledon. This is an example wherein the gene desired to be introduced into the monocotyledon is arranged in a plasmid having the cloned DNA fragment originated from the virulence region of pTiBo542. In Fig. 1, each reference symbol represents the following meaning:

- SP: spectinomycin-resistant gene
- HPT: hygromycin-resistant gene
- NPT: kanamycin-resistant gene
- TC: tetracycline-resistant gene
- IG: intron GUS gene
- BR: right border sequence of T-DNA
- BL: left border sequence of T-DNA
- virB, virC, virG: vir regions originated from super-virulent Agrobacterium tumefaciens A281

ORI: replication origin of ColE1

COS: COS site of  $\lambda$  phage

K: restriction enzyme Kpn I site    H: restriction enzyme Hind III site

5            The gene which is desired to be incorporated into the monocotyledon may be inserted into a restriction site in the T region of the above-described plasmid, and the desired recombinant plasmid may be selected depending on an appropriate selection marker such as drug resistance and the like which the plasmid has. However, if the vector, like pTOK162 shown in Fig. 1, is large and has a number of restriction sites, it is not always easy to insert the desired DNA in the T region of the vector. In such a case, the desired DNA can be inserted in the T region of pTOK162 by utilizing the in vivo homologous recombination (Herrera-Esterella L. et al, 1983; EMBO J. 2:987-995, Horch R. H. et al. Science 1984; 223:496-498) in the cells of Agrobacterium tumefaciens. That is, pTOK162 is preliminarily introduced into Agrobacterium tumefaciens and the plasmid pBR322 (or a similar plasmid) containing the desired gene is further introduced into Agrobacterium tumefaciens. Since pTOK162 has a region homologous with a region of pBR322, the pBR322 derivative containing the desired gene is inserted into pTOK162 by the genetic recombination via the homologous regions. Unlike pTOK162, pBR322 cannot replicate by itself in Agrobacterium tumefaciens. Therefore, pBR322 can only be

alive in Agrobacterium tumefaciens in the inserted form in pTOK162 (the recombined pTOK162 and pBR322 is hereinafter designated "pTOK162::pBR322 derivative"). By selecting the transformants based on the selection

5 markers (such as drug resistance) specific to each of pTOK162 and pBR322 derivative, Agrobacterium tumefaciens transformants containing pTOK162::pBR322 derivative may be obtained. The present inventors made a study by introducing various plasmids into Agrobacterium

10 tumefaciens containing pTOK162, to discover that as the selection marker of the pBR322 derivative, spectinomycin-resistant gene (SP) originated from transposon Tn7 (De Greve H.H. et al., 1981; Plasmid 6:235-248) is suited. Thus, in cases where the desired

15 gene has already been cloned into pBR322, by inserting SP gene into the plasmid, the desired DNA can be inserted in the T region of pTOK162 by homologous recombination in vivo in Agrobacterium tumefaciens. Alternatively, a plasmid containing the DNA from pBR322 and SP gene is

20 first provided, and the desired gene may be inserted into this plasmid. In this case, by utilizing the border sequences of the T region, it is possible to arrange the kanamycin-resistant gene and the desired gene in separate T regions in pTOK162. When plants are transformed using

25 the resistance to kanamycin as a marker, there is a substantial probability that both T regions are introduced, the introduction of the desired gene can be

sufficiently attained. Further, in this case, since both T regions may be inserted into different chromosomes, it may be possible to subsequently segregate the desired gene from the kanamycin-resistant gene.

5           The gene which is desired to be introduced to monocotyledons is not restricted at all and may be any gene which can give a desired character. Examples of the desired gene include herbicide-resistant genes, antibiotic-resistant genes, virus coat protein genes for  
10           giving resistance to the virus, genes related to starch formation in albumen and the like, although the desired genes are not restricted thereto.

          As the host bacterium belonging to genus Agrobacterium, Agrobacterium tumefaciens may preferably  
15           be employed, although not restricted.

          Introduction of a plasmid into the bacterium belonging to genus Agrobacterium such as Agrobacterium tumefaciens can be carried out by a conventional method such as triple cross method of bacteria (Ditta G. et al.,  
20           1980; Proc. Natl. Acad. Sci. USA 77:7347-7351).

          Since the Agrobacterium prepared as mentioned above has a highly virulent DNA originated from pTOK162, transformation of monocotyledons can be attained with a high efficiency.

25           It should be noted that in the method of the present invention, although the gene which is desired to be introduced into the monocotyledon is arranged between

border sequences of the T region as in the prior art, the desired gene may be arranged in the Ti plasmid or in another plasmid in the Agrobacterium. The transformation of the cultured tissue of a monocotyledon by the Agrobacterium may be carried out by merely contacting the cultured tissue with the Agrobacterium. For example, a cell suspension of the Agrobacterium having a population density of  $10^6$  -  $10^{11}$  cells/ml is prepared and the cultured tissue is immersed in this suspension for 3 - 10 minutes. The resulting culture tissue is then cultured on a solid medium for several days together with the Agrobacterium. Alternatively, transformation may be carried out by adding the Agrobacterium to the culture medium of the cultured tissue and continuously culturing the cultured tissue together with the Agrobacterium. Thus, in the method of the present invention, the cultured tissue may be subjected to the transformation without pretreatment such as treating the cultured tissue with an enzyme or injuring the cultured tissue.

After subjecting the cultured tissues to the transformation, it is preferred to select the transformed cells or transformed tissues in the state during dedifferentiation process or in the dedifferentiated state. This can be attained by culturing the treated tissues on a medium containing a plant growth regulator such as an auxin or a cytokinin, which contains a

selectable marker such as hygromycin and an antibiotic against the Agrobacterium, that is employed for the selection by the selection marker. The selected cells or the selected tissues may be subjected to regeneration culture by a known method. By this, plants acquired the desired character by the transformation can be regenerated.

The present invention will now be described by way of examples thereof. It should be noted, however, that the present invention is not restricted to the examples.

#### Example 1

##### (1) Preparation of Sample Cultured Tissues

##### (i) Variety of Rice

Varieties Asanohikari, Tsukinohikari and Koshihikari, which are varieties of japonica rice were selected as samples.

##### (ii) Scutellum and Scutellum Callus

Mature seeds of rice were sterilized by being immersed in 70% ethanol for 1 minute and then in 1% sodium hypochlorite solution for 30 minutes. The seeds were then placed on 2N6 solid medium (inorganic salts and vitamins of N6 (Chu C.C., 1978; Proc. Symp. Plant Tissue Culture, Science Press Peking, pp.43-50), 1g/l of casamino acid, 2 mg/l of 2,4-D, 30 g/l of sucrose, 2 g/l of Gelrite). Scutella were removed from the seeds on Day 4 from the beginning of the culture on 2N6 solid medium and used as "scutellum" samples. On the other hand,

after culturing the mature seeds for about 3 weeks, the formed calli originated from scutella were transferred to 2N6 medium and cultured therein for 4 - 7 days. The resulting calli were used as "scutellum callus" samples.

#### 5 (ii) Shoot Apex Tissue

Mature seeds of rice were sterilized by the above-described method and were placed on 1/2 N6 solid medium (half strength of major inorganic salts and minor salts of N6, vitamins of N6, 1 g/l of casamino acid, 20 g/l of sucrose and 2 g/l of Gelrite). From seedlings on Day 3 after germination, tissues of 2 - 3 mm length containing apex dividing tissues were cut out and used as samples.

#### (iv) Root Segment and Root Callus

15 From the seedlings obtained by the method described in (iii), tip portions of 5 - 10 mm length of the seed roots were cut out and used as "root segment" samples. On the other hand, these radicles were cultured on 2N6 solid medium for about 2 weeks to obtain calli, and these  
20 calli were used as "root callus" samples.

#### (v) Suspended Cultured Cells

The calli originated from scutella were transferred to AA liquid medium (major inorganic salts of AA, amino acids of AA and vitamins of AA (Toriyama and Hinata 1985; Plant Science 41:179-183), MS minor salts (Murashige and Skoog 1962; Physiol. Plant. 15:473-497), 0.5 g/l of casamino acid, 1 mg/l of 2,4-D, 0.2 mg/l of kinetin, 0.1



mg/l of gibberellin and 20 g/l of sucrose) and the cells were cultured therein at 25°C in the dark under shaking of 120 rpm to obtain suspended cultured cells. The medium was replaced with fresh medium every week. (2) Ti

5 Plasmid (Binary Vector)

Hygromycin resistant gene (HPT) and  $\beta$ -D-glucuronidase (GUS) gene were inserted in the T-DNA region of Ti plasmid to obtain the following plasmids:

(i) pIG121 Hm:

10 A plasmid in which the GUS gene containing the first intron of the catalase gene of castor bean and a hygromycin-resistant gene were ligated (Nakamura et al., 1991; Plant Biotechnology II (Extra Issue of GENDAI KAGAKU, pp.123-132), presented from Dr. Nakamura of  
15 Nagoya University).

(ii) pTOK232

1. Insertion of Intron GUS and Hygromycin-resistant Genes to Intermediate Vector pTOK229

The Cla I fragment (2.5 kb) of the  
20 spectinomycin-resistant gene originated from Tn7 were treated with Klenow fragment to blunt the ends. The resulting fragment was inserted in Sma I site of pUC19 to obtain a plasmid pTOK107 (5.2 kb) having ampicillin-resistant and spectinomycin-resistant genes.

25 The obtained pTOK107 was treated with Eco RI and Hind III and the obtained 2.5 kb fragment containing the spectinomycin-resistant gene was ligated to a Eco RI

pTOK162 obtained by inserting virB, virC and virG genes of strongly virulent Agrobacterium tumefaciens A281 into the binary vector was carried out by homologous recombination. That is, since both vectors contain a region originated from an E. coli plasmid pBR322, in the bacterial cells selected by resistances to spectinomycin and kanamycin, only the plasmid generated by recombination of the both plasmids are contained. The plasmid obtained by the fact that the hygromycin-resistant gene and the intron GUS gene were inserted into the super binary vector is called pTOK232 (see Fig. 1).

### (3) Host Agrobacterium tumefaciens

Strains LBA4404 and EHA101 in which T-DNA regions were deleted were used as the host bacteria. Strain LBA4404 has a helper plasmid PAL4404 (having a complete vir region), and is available from American Type Culture Collection (ATCC 37349). Strain EHA101 has a helper plasmid having the vir region originated from a strongly virulent Agrobacterium tumefaciens A281, and is available from Hood E.E. et al., 1986.

The various binary vectors described in (2) were introduced into these two strains of Agrobacterium tumefaciens, and the strains described in the following were used for introducing the genes. The plasmids were introduced into the Agrobacterium strains by triple cross (Ditta G. et al., 1980; Proc. Natl. Acad. Sci. USA 77:

- Hind III fragment (2.7 kb) of pGA482 to obtain pTOK170 (5.2 kb) containing the spectinomycin-resistant gene and has Hind III site and Hpa I site.

A vector pIG221 in which the first intron of catalase of castor bean and GUS gene are ligated to 35S promoter (Ohta S. et al., 1990; Plant Cell Physiol. 31: 805-813, presented by Dr. Nakamura of Nagoya University) was digested with Eco RI and the resultant was treated with Klenow fragment to blunt the ends. To the resultant, a Hind III linker (pCAAGCTTG, code 4660P commercially available from TAKARA SHUZO). A fragment containing 35S promoter and intron GUS was cut out by digesting the resulting vector with Hind III, and the fragment was inserted into the Hind III site of a plasmid pGL2 (J. Paszkowski, obtained from Friedrich Miescher Institute) containing a hygromycin-resistant gene ligated to 35S promoter, to obtain pGL2-IG (7.6 kb). The above-mentioned plasmid pGL2 was obtained by inserting a hygromycin-resistant gene (Gritz L. and Davis J. 1983; Gene 25: 179 - 188) into pDH51 (Pietrazak et al., 1986; Nucleic Acids Research 14: 5857 - 5868). The fragment obtained by treating pTOK170 with Hpa I was ligated to a Pvu II fragment (5.2 kb) of pGL2-IG to obtain pTOK229 (10.1 kb).

2) Insertion into Super Binary Vector pTOK162

Insertion of the desired genes (hygromycin-resistant gene and intron GUS gene) into a super binary vector

7347-7351).

LBA4404 (pTOK232)

LBA4404 (pIG121Hm)

EHA101 (pIG121Hm)

5 (4) Preparation of Suspension of Agrobacterium tumefaciens

Colonies obtained by culturing the Agrobacterium strains on AB medium (Drlica K.A. and Kado C.I. 1974; Proc. Natl. Acad. Sci. USA 71:3677-3681) containing  
 10 hygromycin (50 µg/ml) and kanamycin (50 µg/ml) for 3 - 10 days were collected with a platinum loop and suspended in modified AA medium (same as the composition of the above-described AA medium except that concentrations of sucrose and glucose were changed to 0.2 M and 0.2 M,  
 15 respectively, and that 100 µM of acetosyringone was added, pH 5.2). The cell population was adjusted to  $3 \times 10^9$  -  $5 \times 10^9$  cells/ml and the suspensions were used for inoculation.

(5) Inoculation Conditions

20 The sample tissues were washed with sterilized water and immersed in the above-described suspensions of Agrobacterium strains, respectively, for 3 - 10 minutes. Thereafter, the shoot apex samples were placed on N6S3 solid medium (1/2 N6 major inorganic salts, N6 minor  
 25 salts, N6 vitamins, Chu C.C., 1978, AA amino acids (Toriyama and Hinata 1985), 1 g/l of casamino acid, 0.2 mg/l of NAA, 1.0 mg/l of kinetin and 3 g/l of Gelrite)

containing 100  $\mu$ M of acetosyringone, 10 g/l of glucose and 20 g/l of sucrose. The other tissue samples such as scutellum callus samples were cultured on 2N6 solid medium containing acetosyringone, glucose and sucrose in the same concentrations as mentioned above. The both culture was carried out at 25°C in the dark for 2 - 5 days. The resulting inoculated tissues were then washed with sterilized water containing 250 mg/l of cefotaxime and then continued to be cultured on the respective solid media containing the same concentration of cefotaxime as mentioned above.

(6) Method for Examining GUS Activity

Immediately after the above-mentioned culture with the Agrobacterium strains, the tissues were immersed in 0.1 M phosphate buffer (pH 6.8) containing 0.1% TRITON X-100 at 37°C for 1 hour. After washing off the Agrobacterium strains with phosphate buffer, phosphate buffer containing 0.1 mM of 5-bromo-4-chloro-3-indolyl- $\beta$ -D-glucuronic acid and 20% methanol was added to the tissues. After incubation at 37°C for 24 hours, the number of blue-colored tissues were counted under a microscope and the percentages thereof based on the number of samples are described. In the judgment of the GUS activities of the plants assumed to be transformants after the selection treatment, leaves were collected from the plants and GUS staining was performed in the same manner. If the entire leave or the cut face of the leave

is uniformly colored in blue, the plant was judged to be a positive plant, and if the leave or the cut face of the leave is chimerically stained, the plant was judged to be a chimera plant.

5 (7) Selection of Transformed Cells and Tissues (i)

Shoot Apex

Shoot apices cultured with the Agrobacterium strains for 5 days were cultured on N6S3 medium containing 250 mg/l of cefotaxime for 2 weeks. The grown shoot apex  
10 tissues were transplanted to N6S3 medium containing 40 mg/l of hygromycin and selection of the transformants was carried out.

(ii) Scutellum

Scutella cultured with the Agrobacterium strains for  
15 3 days were cultured on 2N6 medium containing 250 mg/l of cefotaxime for 1 week. Selection of transformed cells were then carried out on 2N6 medium containing 50 mg/l of hygromycin.

(iii) Cultured Tissue (Scutellum Callus)

20 Tissues cultured with the Agrobacterium strains for 3 days were cultured on 2N6 medium containing 250 mg/l of cefotaxime for 1 week. Hygromycin-resistant cultured tissues were selected by culturing the cultured tissues on 2N6 medium containing 50 mg/l of hygromycin for 3  
25 weeks (primary selection). The obtained resistant tissues were further cultured on N6-12 medium (N6 inorganic salts, N6 vitamins, 2 g/l of casamino acid, 0.2

mg/l of 2,4-D, 0.5 mg/l of 6BA, 5 mg/l of ABA, 30 g/l of sorbitol, 20 g/l of sucrose and 2 g/l of Gelrite) containing 50 mg/l of hygromycin for 2 - 3 weeks (secondary selection), and the calli grown on this medium were transferred to a plant regeneration medium N6S3 containing 0, 20 or 50 mg/l of hygromycin. In all of the media used after the culture with Agrobacterium strains, cefotaxime was added to 250 mg/l.

(iv) Suspended Cultured Cells

The cells cultured with the Agrobacterium strains for 5 days were cultured in 2N6 medium containing 250 mg/l of cefotaxime for 1 week, and then the selection of the transformed cells was carried out on 2N6 medium containing 50 mg/l of hygromycin.

(8) Expression of Introduced Genes in the Progeny of Transformants

Seeds of the progeny of the transformants were sown in aqueous 400-fold Homai hydrate (Kumiai Kagaku Inc.) solution containing 70 mg/l of hygromycin and incubated therein at 25°C for 10 days, thereby examining the resistance to hygromycin. Twenty seeds of each plant of the progeny of the transformants were sown and cultured for about 3 weeks. From the obtained seedlings, leaves were collected and examined for the expression of GUS gene.

(9) Analysis of Introduced Genes by Southern Blotting Method

From the primary transformants of varieties Asanohikari and Tsukinohikari, DNAs were extracted by the method of Komari et al. (Komari et al., 1989; Theoretical and Applied Genetics 77: 547-552), and the DNAs were

5 treated with a restriction enzyme Hind III. The resulting fragment was subjected to detection of the introduced genes by Southern blotting analysis using the HPT gene as a probe. The length of the Hind III fragment containing the HPT gene as a probe is about 5.5 kb and

10 the length of the DNA region from the Hind III site in the T-DNA in this region to the L border sequence is about 5.4 kb (Fig. 1). The Southern blotting analysis was carried out in accordance with Molecular Cloning (Sambrook et al., 1989; Cold Spring Harbor Laboratory

15 Press). Two GUS positive plants, two GUS negative plants and two hygromycin-resistant plants were picked up from each of the two lines of the progeny of transformants of Tsukinohikari and were subjected to the Southern blotting analysis in the same manner as mentioned above.

20 (10) Differences in Efficiencies of Introducing Genes Depending on Sample Tissues (Expression of GUS after Culturing with Agrobacterium Strains)

In order to confirm that Agrobacterium can introduce genes into cells of monocotyledons, various tissues of

25 the rice variety Tsukinohikari were treated with Agrobacterium tumefaciens EHA101 having a super-virulent vir region, into which the binary vector (supra)



containing the hygromycin-resistant gene and the GUS gene were introduced, and then the GUS activities were examined. The sample tissues were shoot apices, radicles, scutella, radicle calli, scutellum calli and suspended

5 cultured cells. In cases where the tissues were not treated with the Agrobacterium strain, no tissues exhibited GUS activity indicated by blue color. On the other hand, in cases where the tissues were treated with Agrobacterium tumefaciens EHA101 (pIG121Hm), in all of

10 the tissues except for radicles, expression of GUS was confirmed. The ratio of the number of the tissues showing blue color to the number of treated tissues was the highest in scutellum calli (Table 1). Further, the size of the tissues expressing GUS was also largest in

15 scutellum calli. The tissues exhibited the second highest rate of introduction next to the scutellum calli were shoot apices. Further, while the scutellum calli and the suspension cells which are dedifferentiated tissues of scutella exhibited high introduction rate, the

20 introduction rate in scutella was apparently lower. This suggests that genes are more easily introduced into tissues having high cell-dividing activities.

Table 1

Differences in Efficiency of Introduction of GUS Gene Depending on Sample Material  
(Variety:Tsukinohikari)

Sample Tissue	Number of GUS+ Tissues /Number of Sample Tissues(%)		Size of GUS-stained Portion Based on Treated Tissue
	Non-Treated Group	Treated Group	
Shoot Apex	0/ 30 (0)	109/157 (69)	+++
Root Segment	0/ 20 (0)	0/ 30 (0)	
Root Callus	0/ 30 (0)	24/115 (21)	+
Scutellum	0/ 50 (0)	8/ 89 (9)	+
Scutellum Callus	0/141 (0)	312/395 (79)	+++
Suspension Cells	0/232 (0)	61/247 (25)	++

+ : 1% or less, ++ : 1~10%, +++ : 10% or more

It has been confirmed that the binary vector pIG121Hm used in this experiment does not express GUS gene in Agrobacterium cells because the intron of castor oil plant is inserted in the promoter of the GUS gene (Nakamura et al., 1991). Thus, from the results of the experiments described above in which the expression of the GUS gene after the culturing with Agrobacterium is used as an index, it was confirmed that genes can be introduced into rice cells by Agrobacterium.

#### 10 (11) Differences in Rate of Emergence of Transformed Tissues and Cells Depending on Sample Tissues

Selection of transformed tissues and transformed cells was carried out according to the resistance to hygromycin, using shoot apices, scutella, scutellum calli and suspension cells after the culture with the Agrobacterium strain. As a result, growth of transformants exhibiting resistance to hygromycin were observed in scutellum calli and the suspension cells (Table 2). Further, all of the selected cells expressed the GUS gene. Although the shoot apex tissues exhibited high rate of introduction of GUS gene after the culture with the Agrobacterium strain, after the selection by hygromycin, all tissues died and no tissues resistant to hygromycin were obtained. Although shoot apex is a tissue containing the apical meristem, in order that cells expressing a hygromycin-resistance keep to grow after the treatment for introducing the gene, it is

necessary that the gene be introduced into the limited apical meristem. The reason why no resistant tissues were obtained in spite of the fact that a number of genes were introduced into the shoot apices after the culture with the Agrobacterium strain is thought to be that the probability that the gene is introduced in the vicinity of the apical meristem is low. Further, it is easily assumed that even if a gene is introduced in the vicinity of the apical meristem so that transformed cells are obtained, the possibility that the obtained plants exhibit chimeric properties is high. From these, it is thought that the transformation method utilizing shoot apex, which was reported by Gould et al (1991) has more technical difficulties and less reproducibility than the method utilizing a dedifferentiated tissue such as callus.

While transformed cells were obtained from the scutellum calli and the suspension cells which were originated from scutella of mature seeds, resistant cells were not obtained from the scutellum samples. Although the introduction of the genes was tried by using injured scutella in accordance with the teachings by Raineri et al. (1990), the efficiency of introducing genes was not promoted and no transformed cells were obtained. In contrast, in cases where scutellum calli were used as the samples, transformed cells were obtained with good reproducibility and high frequency, without a treatment

[illegible][illegible]

Table 2  
Differences in Rate of Emergence of Transformed Tissues and Cells Depending on  
Sample Material  
(Variety:Tsukinohikari)

Sample Tissue	Number of Hygromycin-resistant Tissues /Number of Treated Tissues (%)	
	Non-Treated Group	Treated Group
Shoot Apex	0/ 20 (0)	0/ 77 (0)
Scutellum	0/ 30 (0)	0/128 (0)
Scutellum Callus	0/ 50 (0)	169/743 (23)
Suspension Cells	0/250 (0)	22/254 ( 9)

(12) Differences in Efficiencies of Introducing Genes Depending on Varieties of Rice (Expression of GUS after Culturing with Agrobacterium Strains)

There are large differences among varieties about the conditions for establishing cultured cells and for regenerating plants from the cultured cells (Mikami and Kinoshita 1988; Plant Cell Tissue Organ Cult. 12:311 - 314). It is said that Koshihikari is difficult to culture among the Japonica rices. On the other hand, Tsukinohikari employed in the preceding section is relatively easy to culture. When using the transformation method utilizing Agrobacterium, it is practically inconvenient if such differences among varieties exist. In order to clarify this point, the differences in the efficiencies of gene introduction between Koshihikari and Tsukinohikari which have different easiness to culture were examined. The sample tissues employed were scutellum calli and the Agrobacterium tumefaciens strains employed were EHA101(pIG121Hm) and LBA4404(pIG121Hm).

While GUS activity was observed in not less than 90% of calli of Tsukinohikari in each experiment, the GUS activity was observed in Koshihikari at lower rates (Table 3). Thus, in cases where EHA101(pIG121Hm) or LBA4404(pIG121Hm) is used, there is a difference in the introduction efficiency between the varieties.

Table 3

Differences in Rate of Introduction of GUS Gene Depending on Agrobacterium Strain and Rice Variety

		Number of GUS+ Tissues / Number of Treated Tissues (%)		
		Strain		
Variety	Experiment	LBA4404 (pIG121Hm)	EHA101 (pIG121Hm)	LBA4404 (pTOK232)
Tsukinohikari	1	67/70 (96)	78/87 (90)	64/66(97)
Tsukinohikari	2	72/86 (84)	68/73 (93)	82/82(100)
Koshihikari	1	46/135(34)	43/116(37)	124/131(95)
Koshihikari	2	28/107(26)	81/143(57)	102/103(99)



(13) Differences in Efficiencies of Introducing Genes Depending on Agrobacterium Strains (Expression of GUS Gene after Culturing with Agrobacterium Strains)

EHA101(pIG121Hm) has a helper plasmid containing the  
 5 vir region of super-virulent Agrobacterium tumefaciens A281. LBA4404(pIG121Hm) has an ordinary vir region. On the other hand, although the vir region of the helper plasmid in LBA4404(pTOK232) is ordinary, a gene which is a part of the vir region of the super-virulent  
 10 Agrobacterium tumefaciens A281 is contained in the binary vector. This binary vector is originated from pTOK162 and made it possible to transform at a very high rate dicotyledonous species which are difficult to transform (Saito Y. et al., 1992; Theor. Appl. Genet. 83:679-683).  
 15 Thus, there is a possibility that the transformation efficiency is largely influenced by the existence a super-virulent vir region or by the manner of existence thereof. Thus, using the above-described three  
Agrobacterium strains whose vir regions are different,  
 20 the efficiencies of introducing GUS gene were compared. The samples used were scutellum calli of Koshihikari and Tsukinohikari.

Even with LBA4404(pIG121Hm) which does not have a super-virulent vir region, tissues exhibiting GUS  
 25 activities were obtained in both varieties. However, in Koshihikari, the rate was as low as about 30%. With EHA101(pIG121Hm) having the super-virulent vir region in

the helper plasmid, the introduction efficiency in Koshihikari was somewhat higher. With LBA4404(pTOK232) having the super-virulent vir region in the binary vector, GUS activities were observed in not less than 95% tissues even with Koshihikari as with Tsukinohikari (Table 3). Further, as for the area of blue-colored regions in each tissue, the area was the largest with LBA4404(pTOK232), which indicates a high introduction efficiency.

#### 10 (14) Differences in Selection Efficiency Depending on Strains (Hygromycin-resistant Callus)

Using the above-mentioned 3 strains, the selection rates of hygromycin-resistant calli after culturing scutellum calli of Tsukinohikari and Koshihikari with the Agrobacterium strains were compared. As for the rate of emergence of the resistant calli, LBA4404(pTOK232) exhibited the highest rate. No differences about the rate of selection were observed between the varieties (Table 4). With the strains LBA4404(pIG121Hm) and EHA101(pIG121Hm), the rates of selection were low. Especially, with Koshihikari which is difficult to culture, the rate of emergence of hygromycin-resistant calli was as low as about 2%. Thus, it is thought that LBA4404(pTOK232) having a part of the super-virulent vir gene in the binary vector is the best as the Agrobacterium strain used for transforming rice.

Table 4

Differences in Transformation efficiency Depending on Agrobacterium Strain  
(Scutellum Callus)

		Number of Hygromycin-resistant Callus / Number of Treated Callus (%)		
		Strain		
Variety	Experiment	LBA4404 (pIG121Hm)	EHA101 (pIG121Hm)	LBA4404 (pTOK232)
Tsukinohikari	1	91/338 (27)	139/301 (46)	169/305 (55)
Tsukinohikari	2	59/421 (14)	66/425 (16)	110/360 (31)
Tsukinohikari	3		10/521 ( 2)	174/644 (27)
Tsukinohikari	4		20/349 ( 6)	100/349 (29)
Koshihikari	1	6/269 (2)	.	65/283 (23)

(15) Manner of Expressing GUS Gene in  
Hygromycin-resistant Transformants

The thus obtained resistant calli were subjected to secondary selection, and plants were regenerated from the selected resistant calli. A group in which hygromycin was not added to the N6S3 medium for regeneration was provided. In this group, a number of plants which did not exhibit GUS activity or which exhibited GUS activity chimerally emerged. However, in cases where hygromycin was added to the regeneration medium, the number of these plants largely decreased and the number of plants each of which exhibits GUS activity in the whole plant was increased (Table 5, Table 6 and Table 7). In cases where the tissue was not treated with Agrobacterium, no plants which exhibited resistance to hygromycin or GUS activity were obtained. Therefore, the plants each of which exhibited GUS activity in the whole plant, which was regenerated from the hygromycin-resistant callus, are considered as transformants.

Table 5

Expression of GUS Gene in Plants Regenerated from Hygromycin-resistant Calli  
(Variety:Asanohikari)

Resistant Callus	Number of Regenerated Plants	Expression of GUS Gene		
		Stably Positive	Chimera	Negative
1	26	25	1	0
2	8	7	1	0

(Hygromycin was added to culture medium until regeneration of plants.)

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Table 6

Expression of GUS Gene in Plants Regenerated from Hygromycin-resistant Calli  
(Variety:Tsukinohikari)

Sample Strain	Number of Lines		
	Sample Hygromycin -resistant Calli	Calli Yielded Regenerated Plants	GUS Positive Regenerated Plants
LBA4404(pIG121Hm)	3	1	1
EHA101(pIG121Hm)	2 0	1 7	1 0
LBA4404(pTOK232)	2 0	1 5	1 2

(Hygromycin was added to culture medium until regeneration of plants.)

Table 7

Expression of GUS Gene in Plants Regenerated from Hygromycin-resistant Calli  
(Variety:Asanohikari)

Sample Strain	Number of Lines		
	Sample Hygromycin -resistant Calli	Calli Yielded Regenerated Plants	GUS Positive Regenerated Plants
LBA4404(pIG121Hm)	1 9	5	3
EHA101(pIG121Hm)	1 1	4	1
LBA4404(pTOK232)	1 9	1 1	1 1

(Hygromycin was added to culture medium until regeneration of plants.)

#### (16) Ploidy and Fertility of Seeds of Transformants

When cultivated in a green house, the thus obtained transformants exhibited normal growth and morphology and no plants exhibited characteristics of tetraploid or malformation. As for the fertility of the seeds, although some plants exhibited partial infertility or complete infertility, most plants exhibited substantially normal fertility.

#### (17) Expression and Analysis of Introduced Genes in the Primary Transformants and in the Progeny

Fragments obtained by Hind III digesting the whole DNAs in the primary transformants were subjected to detection of the introduced gene by Southern blotting analysis using the HPT gene as a probe. As a result, in all of the tested plants, the existence of the introduced gene in a number of 1 to several copies was confirmed (Table 8 and Table 9). While the size of the Hind III fragment containing the HPT gene in plasmid pTOK232 is 5.5 kb, in all of the tested transformants, a band having a size larger than about 6 kb was observed. This demonstrates that the T-DNA was incorporated into the plant chromosomes. The fact that the size of the detected DNA fragment differed from plant to plant indicates that the site in which the HPT gene was introduced was different from transformant to transformant. Therefore, it was confirmed that the fact that the introduced gene was detected was not due to the

bacteria remaining in the plants.

The resistance to hygromycin of the progeny of the transformants was examined. With the seeds of control plants, substantially no germination was observed or the growth after germination was severely inhibited. In contrast, many of the seeds obtained from the transformants exhibited normal germination and growth (Tables 8 and 9). These hygromycin-resistant plants also expressed the GUS gene. In many lines, concerning the expression of hygromycin resistance and the GUS gene, genetic segregation substantially in accordance with one factor segregation was observed. As for transformant lines 1-2 and 3-2 of Asanohikari in Table 8, from the segregation ratio, existence of 2 or more factors of introduced genes is expected. The results of Southern blotting analysis were also compatible with the two factor segregation. In the transformant 2-1 in Table 8, existence of two copies of the introduced genes was confirmed. One of these bands represents a fragment shorter than 5 kb, so that it is assumed that the T-DNA of an incomplete form was incorporated. It is assumed that because of this, this transformant exhibited segregation like one factor segregation about the hygromycin resistance in the progeny.

As shown in Table 9, many of the transformants of Tsukinohikari exhibited one factor segregation about the hygromycin resistance and GUS gene in the progeny.



However, Southern blotting analysis of the primary transformant revealed that most of the transformants contained a plurality of copies of the gene although some of them contained a single copy of the gene. The progeny of the transformant 18a which contained one copy of the introduced gene and the progeny of the transformant 16c which contained two copies of the introduced gene were subjected to Southern blotting analysis. Two plants each of the GUS positive, GUS negative and hygromycin-resistant plants of each line were subjected to the analysis. As a result, in all of the plants except for the GUS negative plants, the same bands as detected in the plants of the primary transformants were detected. Therefore, it was shown that the introduced genes were inherited to the subsequent generations. As for the line 16c having two copies of the introduced gene, the fact that both the progeny of GUS positive plants and hygromycin-resistant plants had the same two copies of the introduced genes suggests that a plurality of genes were incorporated into the same chromosome or the same locus.

These results indicate that the genes introduced into rice plants by Agrobacterium were incorporated in the nuclei of the plant cells and were inherited to the subsequent generations in accordance with the Mendel's laws.

Table 8

Number of Copies of Introduced Genes in Transformants Determined by Southern Blotting Analysis and Expression of Introduced Gene in the Progeny of Transformants (Variety:Asanohikari)

Transformants	Number of Copies of Introduced Gene	Number of Plants of the Progeny of Transformants			
		Resistance to Hygromycin		Expression of GUS	
		Resistant	Sensitive	Positive	Negative
Control	—	0	60	0	20
1 - 2	2	30	0	19	1
2 - 1	2*	64	26	13	5
3 - 2	2	59	1	19	1

\*In one of the two copies of the introduced genes, the restriction fragment was short, so that the introduced gene was incomplete.

Table 9

Number of Copies of Introduced Genes in Transformants Determined by Southern Blotting Analysis and Expression of Introduced Gene in the Progeny of Transformants (Variety: Tsukinohikari)

Transformants	Number of Copies of Introduced Gene	Number of Plants of the Progeny of Transformants			
		Resistance to Hygromycin		Expression of GUS	
		Resistant	Sensitive	Positive	Negative
Control	—	0	60	0	20
1	1	46	26	15	5
2 a	2	33	18	13	5
2 b	2	31	9	15	5
3	2	29	10	16	3
4 a	3	22	21	13	7
4 b	3	48	11	16	3
5 a	3	26	13	17	3
5 b	3	36	14	17	3
5 c	3	24	9	17	2
6	2	47	13	—	—
7	1	56	20	14	5
8	4	45	22	—	—
9	1	52	18	18	2
10	4	53	10	—	—
11	2	75	15	18	2
12	3	44	7	14	6
13 a	2	33	18	15	5
13 b	2	32	8	13	7
14 a	1	72	20	15	5
14 b	1	26	14	10	10
15	1 ~ 2	22	7	12	8
16 a	2	31	10	15	2
16 b	2	32	8	14	3
16 c *	2	69	24	13	7
17	6	89	41	—	—
18 a *	1	35	5	15	5
18 b	1	70	20	10	10
19	2	47	13	—	—

\* Introduced genes in plants of the next generation were analyzed by Southern blotting analysis.

Example 2

## (1) Maize Varieties

Maize varieties A188, F1 (A188 x Black Mexican Sweet), F1 (A188 x B73Ht), F1 (B73Ht x A188) and F1 P3732 were selected as the sample materials. The varieties of A188, Black Mexican Sweet and B73Ht were obtained from National Institute of Agrobiological Resources, Ministry of Agriculture, Forestry & Fisheries, and P3732 was obtained from IWATA RAKUNOU KYODOKUMIAI.

## 10 (2) Preparation of Tissues in the Vicinity of Growth Point

Mature seeds were immersed in 70% ethanol for 1 minute and in 1% sodium hypochlorite for 5 minutes. The seeds were then washed three times with sterilized water and were placed on LS solid medium (inorganic salts and vitamins of Linsmaier and Skoog; Linsmaier, E. and Skoog, F. 1965; Physiol. Plant 18: 100 - 127, 100 mg/l of casamino acid, 700 mg/l of proline, 20 g/l of sucrose and 2.3 g/l of Gelrite). After culturing the seeds at 25°C in the dark for 4 days, tissues with a length of about 0.1 x 0.3 mm containing the apex dividing tissues were cut out and used as samples.

## (3) Preparation of Calli Originated from Immature Embryos

25 Immature embryos were placed on LSD1.5 solid medium (inorganic salts and vitamins of Linsmaier and Skoog, 100 mg/l of casamino acid, 700 mg/l of proline, 1.5 mg/l of

2,4-D, 20 g/l of sucrose and 2.3 g/l of Gelrite). After culturing the embryos for 3 weeks, the formed calli originated from scutella were collected and used in the subsequent experiments.

5 (4) Strains of Agrobacterium

Among the strains of Agrobacterium described in Example 1, LBA4404 (pTOK232) and EHA101 (pIG121Hm) were used.

(5) Preparation of Suspensions of Agrobacterium Strains

10 Colonies of the Agrobacterium strains obtained by culturing the Agrobacterium strains on AB medium containing hygromycin (50 mg/l) and kanamycin (50 mg/l) for 3 - 10 days were collected using a platinum loop and the cells were suspended in the modified AA medium  
15 described in Example 1. The cell population was adjusted to  $3 \times 10^9$  -  $5 \times 10^9$  cells/ml and the resultants were used for inoculation.

(6) Conditions for Inoculation to Tissues in the Vicinity of Growth Point and for Culturing

20 After piercing the cut out tissues with a glass needle, the sample tissues were immersed in the above-described suspensions of the Agrobacterium strains for 3 - 10 minutes. The tissues were then transferred to modified LS solid medium (inorganic salts of Linsmaier  
25 and Skoog, vitamins of Murashige and Skoog; Murashige, T. and Skoog, F. 1962; Physiol. Plant. 15:473-497, 0.1 mg/l of kinetin, 1.0 mg/l of casamino acid and 2.3 g/l of

Gelrite) and were cultured at 25°C under illumination for 2 - 3 days. Thereafter, the tissues were washed with sterilized water containing 250 mg/l of cefotaxime and then continued to be cultured on LS solid medium containing the same concentration of cefotaxime.

#### (7) Conditions for Inoculation to Calli and for Culturing

The calli were immersed in the above-described Agrobacterium suspensions for about 5 minutes and the resulting calli were transferred to 2N6 solid medium containing acetosyringone described in Example 1 at 25°C in the dark for 3 days to carry out the culture with the Agrobacterium strains. The calli were washed with sterilized water containing 250 mg/l of cefotaxime and then continued to be cultured on LSD1.5 solid medium containing the same concentration of cefotaxime and 30 mg/l of hygromycin, thereby carrying out the selection of transformed calli.

#### (8) Method for Examining GUS Activity

The shoot apex tissues and the calli immediately after the culture with the Agrobacterium strains and the shoot apex tissues and the calli which were continuously cultured after the culture with the Agrobacterium strains were examined for their GUS activities by the method described in Example 1.

#### (9) Introduction of Genes into Shoot Apex Tissues

In order to confirm that transformation employing

the growth point tissues (shoot apex tissues) reported by Gould et al (Gould J. et al., 1991; Plant Physiol.

95:426-434) can be attained, isolated shoot apex tissues were treated with the above-described Agrobacterium

5 tumefaciens strain EHA101(pIG121Hm), and the GUS

activities of the grown plants were examined. While

expression of the GUS gene was not observed in the

tissues which were not treated with the Agrobacterium

strain, expression of the GUS gene was observed in the

10 spots pierced with the needle in the tissues which were

treated with the Agrobacterium strain. The plants

obtained by culturing the tissues were tested for GUS

activities. However, no plants exhibited GUS activity.

The vicinity of the growth point is a very small tissue,

15 so that it is not easy to pierce the needle into the very

small tissue to infect Agrobacterium. The results of

this experiment show that the transformation by infecting

the vicinity of the growth point with Agrobacterium

requires high skill in cutting out and piercing the

20 growth point and the like.

Table 10

Introduction of Gene into Maize Shoot Apex Tissue

Experiment	Number of Sample Tissues	Number of Tissues whose shoot Apices Extended	Number of Plants Obtained	Number of Plants in which GUS was Expressed
1	2 4	9	2	0
2	1 6	8	6	0
3	1 7	1 3	5	0
4	1 4	1	0	0
5	4 5	1 4	7	0
6	3 2	1 4	8	0
7	3 0	7	1	0

Sample variety was P3732 in all experiments.



(10) Differences in Efficiencies of Gene Introduction  
Depending on Maize Varieties and on Strains

In any of the varieties tested, expression of the GUS gene was observed at a high frequency. No  
5 differences were observed between the EHA101(pIG121Hm) and LBA4404(pTOK232) (Table 10). The size of the stained area with respect to the entire callus was not less than 10% in many calli, so that the expression of the gene was observed in wide range of cells. The binary vectors  
10 pIG121Hm and pTOK232 used in this experiment do not express GUS gene in Agrobacterium cells because the intron of castor bean is inserted in the promoter of the GUS gene. Therefore, the expression of the GUS gene observed in the maize calli indicates that gene  
15 introduction was carried out by the Agrobacterium strains with high frequency. After the culture with the Agrobacterium strains, from some of the sample calli, compact and knot-like calli grown. Since the grown cells exhibited expression of the GUS gene, it is thought that  
20 these cells are transformed cells. These compact and knot-like transformed calli can be regenerated to plants according to the method of Lupotto et al (Lupotto, E. and Lusardi, M.C. 1988; Maydica XXXIII:163-177).

Table 11

Efficiency of Introduction of GUS Gene into Maize Calli

Variety	Strain	Number of GUS <sup>+</sup> Calli / Number of Treated Calli (%)
A188	1	32/35 (91)
A188	1	34/34 (100)
A188 × BMS	1	41/49 (84)
A188 × B73	1	35/42 (83)
A188	2	39/40 (98)
A188	2	40/40 (100)
A188 × BMS	2	38/40 (95)
A188 × B73	2	31/40 (78)
B73 × A188	2	29/35 (83)

BMS : Black Mexican Sweet

Strain 1 : EHA101(pIG121Hm), 2 : LBA4404(pTOK232)

Example 3 Transformation of Rice Cultured Tissue  
During Dedifferentiation

(1) Variety of Rice

TSUKINOHKARI, which is a variety of japonica rice  
5 and which is also used in Example 1 was employed.

(2) Super Binary Vector pTOK233

A super binary vector pTOK233 was constructed by a  
process similar to the construction process of pTOK232,  
as shown in Fig. 2, from the above-described pGL2-IG and  
10 pTOK162.

(3) The obtained super binary vector pTOK233 or the  
above-described binary vector pIG121Hm was introduced  
into two strains of *Agrobacterium tumefaciens* by the same  
method as used in Example 1 to obtain the following three  
15 *Agrobacterium tumefaciens* strains.

LBA4404 (pIG121Hm)

EHA101 (pIG121Hm)

LBA4404 (pTOK233)

All of these strains have intron GUS and hygromycin-  
20 resistant gene in the T-DNA region.

(4) Embryos of Mature Seeds

From mature rice seeds cultured on LS2.5 medium (LS  
basal medium supplemented with 2.5 mg/l of 2,4-  
dichlorophenoxyacetic acid) for 5 days (4 days post-  
25 germination), embryos (3-5 mm size) containing scutella  
were excised. The embryos were washed with sterilized  
water and immersed in a cell suspension of *Agrobacterium*

as in Example 1. The embryos were then placed on 2N6 medium containing acetosyringone and cultured at 25°C for 3 days in the dark. Expression of GUS gene was checked using X-gluc as in Example 1. The number of embryos which exhibited GUS expression on the scutella was counted.

#### (5) Embryos of Immature Seeds

Immature seeds at 10-14 days after flowering were sterilized and immature embryos (1.5-2.0 mm size) were excised. The embryos were sterilized by a conventional method and cultured on 2N6 medium for 2 days. The germinated buds were excised and immersed in *Agrobacterium* suspension as in Example 1. The embryos were then placed on 2N6 medium containing acetosyringone and cultured at 25°C for 3 days in the dark.

#### (6) Selection of Resistant Cells and Regeneration

The embryos after the culture with *Agrobacterium* were placed on 2N6 medium containing 50 mg/l of hygromycin and 250 mg/l of cefotaxime and cultured at 25°C for 3 weeks under illumination. The number of the tissues from which calli exhibiting uniform GUS expression were formed was counted. The selected calli were placed on N6-7 medium containing 100 mg/l of hygromycin and 250 mg/l of cefotaxime and cultured at 25°C for 10 days under illumination. The selected calli were placed on N6S3 medium containing 50 mg/l of hygromycin and 250 mg/l of cefotaxime and cultured at

25°C for 40 days under illumination. Using leaves of the obtained regenerated plants, expression of GUS gene was checked using X-gluc as in Example 1.

(7) Results

5 The results are shown in Tables 12-14 below.

Table 12 GUS Expression after Culturing with *Agrobacterium* (embryos derived from mature seeds)

Sample Strain	Number of Sample Embryos	Number of GUS <sup>+</sup> Embryos
EHA101	50	8
(pIG121Hm)	61	11
LBA4404	53	16
(pTOK233)	60	32

Table 13 Results of Transformation (embryos derived from mature seeds)

Sample Strain	Number of Sample Tissues	Number of Lines		
		Hygromycin-resistant and GUS <sup>+</sup> Calli	Calli Yielded Regenerated Plants	GUS <sup>+</sup> Regenerated Plants
EHA101	48	0	0	0
(pIG121Hm)	78	2	0	0
LBA4404	50	1	1	1
(pTOK233)	80	8	5	5

Table 14 Results of Transformation (embryos derived from immature seeds)

Sample Strain	Number of Sample Tissues	Number of Lines		
		Hygromycin-resistant and GUS <sup>+</sup> Calli	Calli Yielded Regenerated Plants	GUS <sup>+</sup> Regenerated Plants
LBA4404 (pIG121Hm)	65	10	4	3
EHA101 (pIG121Hm)	67	15	6	6
	41	8	3	3
LBA4404 (pTOK233)	65	41	28	25
	40	28	17	13

As can be seen from Table 12, with the embryos derived from mature seeds, the number of embryos which showed GUS expression was much larger in the group treated with LBAA4404(pTOK233). As can be seen from Table 13, hygromycin-resistant calli were scarcely obtained in the group treated with EHA101(pIG121Hm) and no regenerated plants were obtained in this group. In contrast, in the group treated with LBA4404(pTOK233), regenerated plants were obtained even though the frequency was low. Most of the regenerated plants expressed GUS and so they are thought to be transformants.

As can be seen from Table 14, with the immature embryos, transformants were obtained in all groups irrespective of the strain used for transformation. However, the transformation efficiency was prominently high when LBA4404(pTOK233) was used.

Thus, the embryo tissues cultured in a dedifferentiation-inducing medium for several days were able to be transformed via *Agrobacterium*. For such transformation, a super binary vector such as pTOK233 contained in the strain LBA4404(pTOK233) gives prominently high transformation efficiency and so is very useful for this purpose.

## CLAIMS

1. A method for transforming a monocotyledon,  
comprising contacting a cultured tissue of said  
monocotyledon during dedifferentiation thereof obtained  
5 by culturing an explant on a dedifferentiation-inducing  
medium for less than 7 days with a bacterium belonging to  
the genus *Agrobacterium* containing a super binary vector  
having the virulence region of Ti plasmid pTiBo542  
contained in *Agrobacterium tumefaciens* A281, left and  
10 right border sequences of T-DNA of a Ti plasmid or an Ri  
plasmid of a bacterium belonging to the genus  
*Agrobacterium*, and a desired gene located between said  
left and right border sequences.
2. The method according to claim 1, wherein said  
15 virulent region in said super binary vector comprises  
*VirB* and *VirG* regions.
3. The method according to claim 1, wherein said  
explant is an immature tissue.
4. The method according to claim 3, wherein said  
20 immature tissue is an immature embryo.
5. The method according to any one of claims 1-4,  
wherein said monocotyledon is a plant belonging to the  
family Gramineae.
6. The method according to claim 5, wherein said  
25 monocotyledon is rice.
7. The method according to any one of claims 1-4,  
wherein said bacterium belonging to the genus

*Agrobacterium* is *Agrobacterium tumefaciens*.

8. The method according to any one of claims 1-4,  
wherein said cultured tissue is transformed by contact  
with a suspension of said *Agrobacterium* having a cell  
5 population of  $10^6$  to  $10^{11}$  cells/ml.

9. The method according to any one of claims 1-4,  
further comprising a step of selecting a transformed cell  
or a transformed tissue during dedifferentiation or in a  
dedifferentiated state, after subjecting said cultured  
10 tissue to transformation.

10. The method according to any one of claims 1-4,  
wherein said cultured tissue has the ability to  
regenerate a normal plant.

11. The method according to claim 8, wherein said  
15 cultured tissue is contacted with said suspension of  
*Agrobacterium* for 3-10 minutes, and then cultured on a  
solid medium for several days together with said  
*Agrobacterium*.

12. The method according to any one of claims 1-4,  
20 wherein said virulence region is originated from a super  
binary vector pTOK162.

13. A method for transforming a monocotyledon,  
comprising contacting a cultured tissue of said  
monocotyledon during dedifferentiation thereof obtained  
25 by culturing an explant derived from an immature tissue  
on a dedifferentiation-inducing medium for less than 7  
days with a bacterium belonging to the genus



*Agrobacterium* containing a desired gene and containing a vector having the virulence region of Ti plasmid contained in *Agrobacterium tumefaciens*.

14. The method according to claim 13, wherein said  
5 immature tissue is an immature embryo.

15. The method according to claim 13 or 14, wherein said monocotyledon is a plant belonging to the family Gramineae.

16. The method according to claim 15, wherein said  
10 monocotyledon is rice.

## ABSTRACT

A method for transforming a monocotyledon by which the time required from transformation to regeneration of a plant is shorter so that the frequency of emergence of mutants is smaller than the conventional methods, which may be generally applied even to the plants for which the regeneration method from a protoplast to a plant has not been established, and with which the preparation of the material to be subjected to the method is easy. That is, the present invention provides a method for transforming a monocotyledon, comprising contacting a cultured tissue of said monocotyledon during dedifferentiation thereof obtained by culturing an explant on a dedifferentiation-inducing medium for less than 7 days with a bacterium belonging to the genus *Agrobacterium* containing a super binary vector having the virulence region of Ti plasmid pTiBo542 contained in *Agrobacterium tumefaciens* A281, left and right border sequences of T-DNA of a Ti plasmid or an Ri plasmid of a bacterium belonging to the genus *Agrobacterium*, and a desired gene located between said left and right border sequences.



FIG. 1

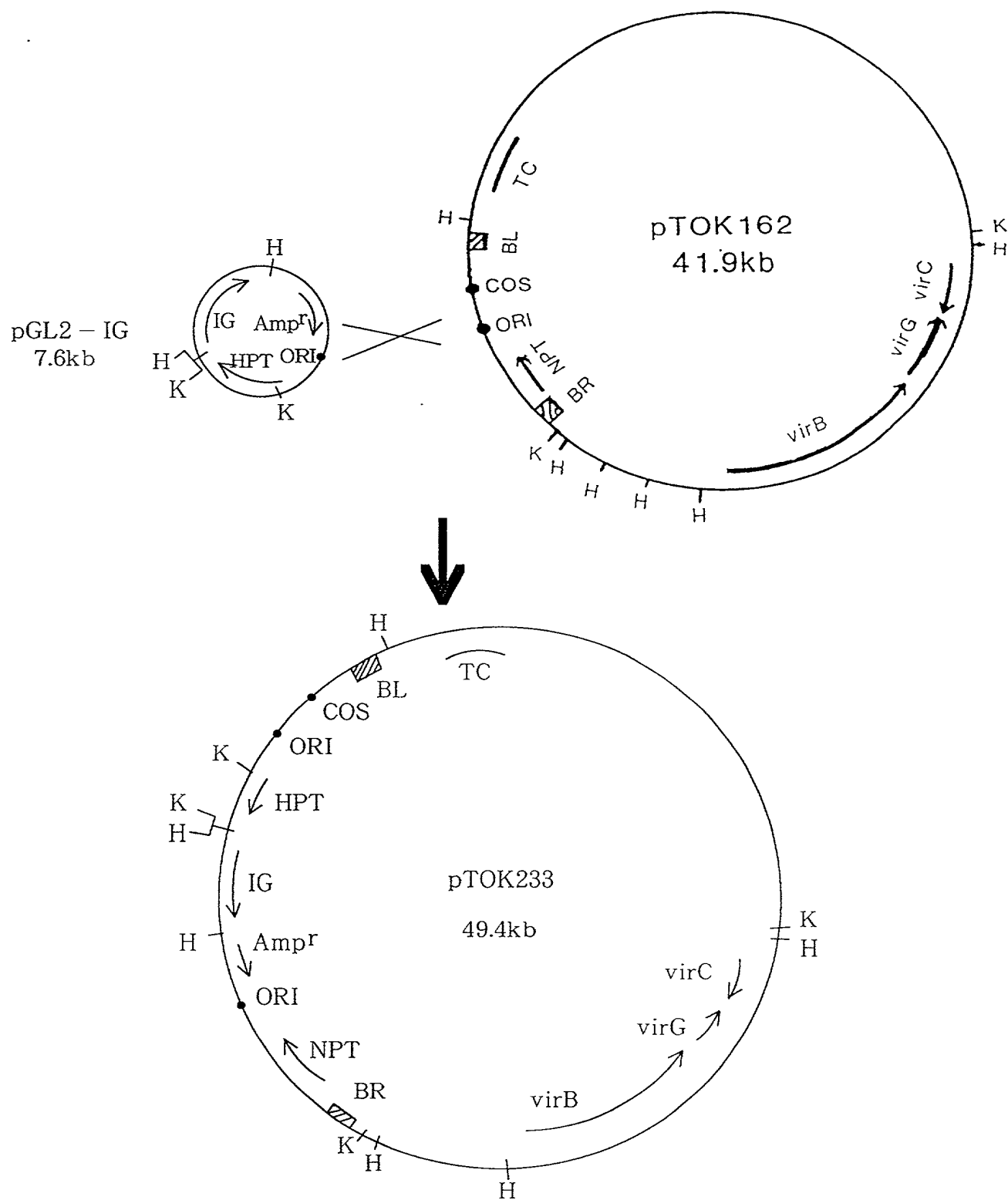


Fig. 2

COMBINED DECLARATION AND POWER OF ATTORNEY  
FOR PATENT APPLICATION

As a below named inventor, I hereby declare that: my residence post office address and citizenship are as stated next to my name; that I verily believe that I am the original, first and sole inventor (if only one inventor is named below) or a joint inventor (if plural inventors are named below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: \*"Method for Transforming Monocotyledons"

the specification of which is attached hereto unless one of the following boxes is checked:

☐ The Specification was filed on \_\_\_\_\_ and was assigned Serial No. \_\_\_\_\_ and was amended on \_\_\_\_\_  
☐ was filed as PCT international application number \_\_\_\_\_ on \_\_\_\_\_ and was amended under PCT Article 19 on \_\_\_\_\_ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I do not know and do not believe the same was ever known or used in the United States of America before my or our invention thereof, or patented or described in any printed publication in any country before my or our invention thereof, or more than one year prior to this application, that the same was not in public use or on sale in the United States of America more than one year prior to this application, that the invention has not been patented or made the subject of an inventor's certificate issued before the date of this application in any country foreign to the United States of America on an application filed by me or my legal representatives or assigns more than twelve months prior to this application, and that no application for patent or inventor's certificate on this invention has been filed in any country foreign to the United States of America prior to this application by me or my legal representatives or assigns, except as follows:

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below:

Prior Foreign Application(s)

Priority Claimed

204464/92      Japan      07/07/92  
(Number)      (Country)      (Month/Day/Year Filed)      Yes

All Foreign Applications, if any, for any Patent or Inventor's Certificate Filed More Than 12 Months Prior To The Filing Date of This Application:

Country      Application No.      Date of Filing (Month/Day/Year)

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

08/193,058      05/03/94      pending  
(Application Serial No.)      (Filing Date)      (Status-patented,  
pending, abandoned)

I hereby appoint the following attorneys to prosecute this application and/or an international application based on this application and to transact all business in the Patent and Trademark Office connected therewith and in connection with the resulting patent based on instructions received from the entity who first sent the application papers to the attorneys identified below, unless the inventor(s) or assignee provides said attorneys with a written notice to the contrary:

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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